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Testosterone Titers of Immature Loggerhead Sea Turtles (*Caretta caretta*) Incidentally Caught in the Central Mediterranean: A Preliminary Sex Ratio Study

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As do many other reptiles, at least six of the seven living marine turtle species demonstrate temperature-dependent

sex determination (see Janzen and Paukstis, 1991, for a review). Apart from being a complex theoretical topic (e.g., see Ewert and Nelson, 1991), temperature-dependent sex determination is also of great relevance to conservation practice, and failure to appreciate the phenomenon may represent a factor increasing the vulnerability of these threatened species, since various human activities (including conservation programs) may unintentionally alter the normal hatchling sex ratio (Mrosovsky and Yntema, 1980). Moreover, since each nesting site may be different from others as regards to thermal (and hence sex-determining) characteristics, a decrease in site availability due to human activity may lead to less thermal variability and this may result in a different overall sex ratio. A knowledge of sex ratio values and their possible temporal changes is of fundamental interest for marine turtle conservation.

In the Mediterranean Sea the serious threats to the populations of *Caretta caretta* and their subsequent decline (see Groombridge, 1990, for a review), make it particularly urgent to include sex ratio dynamics, as well as the other demographic factors influencing population structure, in the planning of conservation strategies. The obvious first step is to investigate the natural sex ratio of loggerheads in the Mediterranean, as no data have been available up to now.

The greatest difficulty in such a study is to have adequate study material. Direct capture of turtles from the wild is difficult in areas where their populations are low and involves major financial commitment. A possible alternative is to collect turtles caught in fisheries as non-target species.

This work undertakes both to provide sex ratio estimates for *Caretta caretta* in the Mediterranean for the first time, and to investigate the possibility of taking advantage of the catch effort normally conducted by professional fishermen.

Materials and Methods. — Adult turtles are not the ideal target for this kind of investigation, in that males and females may have different reproductive behaviors and perhaps also different reproductive cycles (Wibbels et al., 1990). This, together with different migration patterns between feeding, wintering, and breeding sites, may result in the adult sex ratio within a limited zone being unrepresentative of the population as a whole. Utilizing immature and subadult turtles avoids sampling errors of this kind. Furthermore, juveniles, representing a condensation of many cohorts, are a valuable source of insight into demographic structure and, hence, about the productivity of the future adult population.

Immature loggerheads do not show external sexual dimorphism, and the most non-invasive and simple sexing method available at present is based on the evaluation of the levels of testosterone in the blood (Wibbels et al., 1987b).

The 70 specimens of *Caretta caretta* examined in this study were captured within a range of 180 km off Lampedusa Island, Italy (12°35' E; 35°31' N) between 30 June and 20 September in 1991, 1992, and 1993.

This study area, in particular the Gulf of Gabès, is thought to be an important wintering area (Margaritoulis, 1988a; Laurent et al., 1990) and foraging zone (Argano et al.,

1992; Laurent and Lescure, 1994) for loggerheads. This, together with the great mobility of immatures observed within the Mediterranean (Argano et al., 1992), renders it probable that this immature population represents a mix of turtles from several nesting sites, and therefore makes the area itself one of the most suitable in the Mediterranean for sex ratio studies of sea turtles.

All turtles were captured incidentally during the normal activities of professional fishing boats equipped with longlines or trawl-nets, then carried to Lampedusa harbor as part of a tagging program (see Argano et al., 1992).

On the basis of known data about the minimum size of *Caretta caretta* females nesting in the Mediterranean (Margaritoulis, 1982, 1988b), only those specimens with a carapace length less than 70 cm (SCCL, standard curved carapace length) were considered as immatures, with our sample ranging from 29 to 65 cm SCCL.

Blood samples were drawn upon arrival of the turtles at the harbor. In some cases, blood was taken from rehabilitated captive turtles before release. The elapsed time between capture and bleeding was divided into 24-hr classes from 0–24 hrs to 120–144 hrs.

Blood was obtained from the cervical sinus (Owens and Ruiz, 1980) and stored with ice in an insulated bag until centrifugation (max. 2 hrs). The serum obtained was stored at -20°C until September of the corresponding year, then at -70°C until analysis.

Testosterone titers were determined by a radioimmunoassay method similar to that used in analogous work (e.g., Wibbels et al., 1987b). Tritiated testosterone (Amersham) was diluted with tris/gel buffer to yield 6000 cpm per 200 μl . Testosterone antiserum (Sigma) was diluted with tris/gel buffer to obtain 40% of binding of the total tritiated testosterone. A 500- μl aliquot for each sample was extracted with 6 ml of diethyl ether. The extraction efficiency was 80%. The ether phase was dried under nitrogen, then reconstituted with 400 μl of tris/gel buffer and incubated for 12 hrs at 4°C . Each sample was then divided into two aliquots of 200 μl each. Two sets of tubes were prepared: one with 200 μl of testosterone at known concentrations (ranging from 1.9 pg/

ml to 1000 pg/ml) (standards obtained from Sigma), and one with the sample aliquots; 200 μl of tritiated testosterone and 200 μl of antiserum were added to all tubes, which were then incubated for 12 hrs at 4°C . After incubation, unbound tritiated testosterone was separated adding to each tube 200 μl of dextran-coated charcoal (1 g Norit A charcoal, 0.1 g Dextran per 350 ml tris/gel buffer). The tubes were then vortexed, incubated for 10 min at 4°C , and centrifuged for 10 min at $1500 \times g$ in a refrigerated centrifuge ($4-8^{\circ}\text{C}$). The supernatant and 5 ml of scintillation cocktail were poured together into vials and counted for 2 min in a scintillation spectrometer. The significance level for all tests was $\alpha = 0.05$.

Results. — The exact testosterone titer of two turtles was unknown, being above our limits of measurement (> 1000 pg/ml). The frequency distribution for the other 68 specimens is shown in Fig. 1, which demonstrates an apparent bimodal pattern.

No significant differences were found in value distributions between annual groups (1991, 1992, 1993) (Kruskal-Wallis test; two-tailed; $KW = 1.56$; $n = 22, 19, 27$), or between animals captured by longlines (injured by hooks) or by other methods (Mann-Whitney test; two-tailed; $U = 395$; $n = 53, 15$). As far as possible differences between turtles grouped according to interval between capture and bleeding, no significant differences were found either between ≤ 24 hrs and > 24 hrs or between ≤ 48 hrs and > 48 hrs (Mann-Whitney test; two-tailed; $U = 453$; $n = 22, 46$; $U = 408$; $n = 52, 16$).

Discussion. — Previous works have shown, through confirmatory laparoscopic examination of gonads, that immature males and females of *Caretta caretta* in eastern Florida, USA, have different ranges of testosterone titers: ≤ 31 pg/ml for females and > 40 pg/ml for males (Wibbels et al., 1987b; Wibbels et al., 1991). Applying these threshold values for sex ranges (and ignoring possible interpopulation differences in testosterone levels), the 70 specimens we studied may include 11 females (≤ 31 pg/ml), 5 unknowns (31–40 pg/ml) and 54 males (≥ 40 pg/ml). However, further considerations lead us to question this interpretation.

We are concerned that differences in levels of stress may have affected our results. The specimens studied by

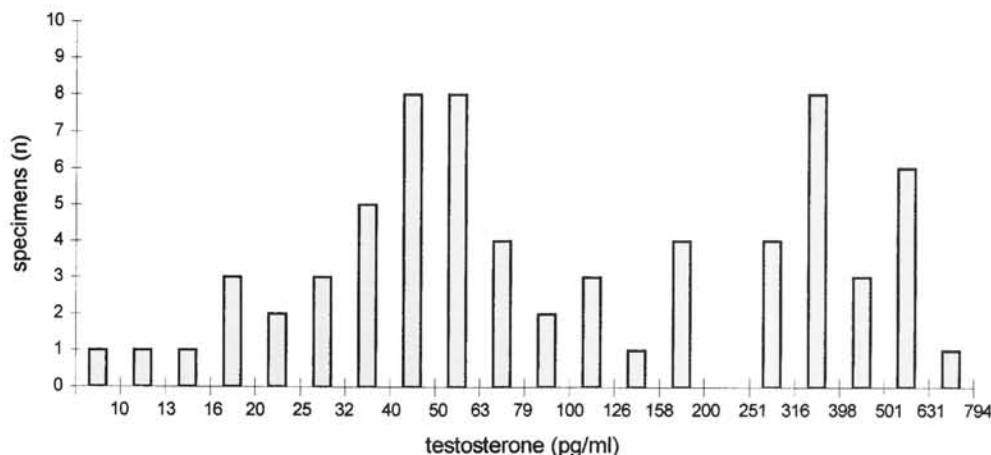


Figure 1. Testosterone titers (on a logarithmic scale) of the 68 specimens with values < 1000 pg/ml (two specimens had values > 1000 pg/ml; see text).

Wibbels et al. (1987b) were bled within ca. 30 min of capture, but our specimens were bled after many hours. Wibbels et al. (1987b), who considered the male range as > 76 pg/ml, reported that two out of four immature females showed increases in testosterone titers 12–16 hrs after capture; the value of one of these reached 53 pg/ml, outside the expected female range. Furthermore, one out of the four groups used in that study had more specimens in the range of 31–76 pg/ml than the other groups (one-tailed; $\chi^2 = 4.78$, $df = 1$, $p < 0.05$; $n = 272$), probably because that group was the only one bled up to 6 hrs after capture. All these clues suggest that the great stress suffered by our study animals may have increased their testosterone titers. Using females as an example, these titers, at first in the normal range (≤ 31 pg/ml), could have risen to reach the unknown range (31–40 pg/ml) and male range (> 40 pg/ml). The frequency distribution of testosterone titers (Fig. 1) suggests that the values of most of the females shifted to a modal zone of around 50 pg/ml.

The observed frequency distribution of values (Fig. 1) appears bimodal, which is what is expected in a case of two sexes having different ranges of values. Thus, it is plausible that the lower mode belongs to females (ca. 50 pg/ml), while the well-separated upper mode belongs to males (ca. 400 pg/ml). The maximum male testosterone titer in the 272 turtles sampled by Wibbels et al. (1987a) was 545 pg/ml, while in the present sample of 70 turtles 7 specimens had values considerably higher (> 1000 pg/ml in two cases).

Assuming that stressed males did not sustain a decrease in testosterone titer, a conservative and plausible view is that those specimens with very high values (≥ 276 pg/ml, represented in Fig. 1 by the columns > 251 pg/ml, and also including the two specimens with values > 1000 pg/ml) were males ($n = 24$), and that those specimens with very low values (≤ 31 pg/ml, represented in Fig. 1 by the columns < 32 pg/ml) were females ($n = 11$) with values remaining in their natural range. In regard to the possible stress-induced increase of female testosterone titers (on the basis of the observed mode of 50 pg/ml), a hypothetical approach might be to consider as females those specimens with values ≤ 68 pg/ml (represented in Fig. 1 by the columns < 79 pg/ml; $n = 36$). As this range overlaps with the male range (> 40 pg/ml, as determined by Wibbels et al., 1991), a few specimens (ca. 6, according to the proportion found by Wibbels et al., 1987b) would need to be subtracted as potential males with values remaining in this range and conservatively considered as "uncertain," together with those specimens with values between 68 and 276 pg/ml (range: 79–190 pg/ml, represented in Fig. 1 by the columns 79–200 pg/ml; $n = 10$).

Table 1. Three different analysis criteria for predicting possible sex ratios of observed testosterone titers in 70 juvenile *Caretta caretta* from the Central Mediterranean.

Criteria	Females		Males		Unknown		Sex Ratio M : F
	n	%	n	%	n	%	
Wibbels et al., 1987b	11	16	54	77	5	7	4.9
Conservative	11	16	24	34	35	50	2.2
Hypothetical	30	43	24	34	16	23	0.8

These different analysis criteria for predicting possible sex ratios in this sample are summarized in Table 1.

Importantly, these results are to be considered preliminary only and need confirmatory validation through examination of specimens of known sex from the same population. Given the approach used, the values also represent a cautious estimate as regards different radioimmunoassay methods as an alternative explanation of the apparent shift of testosterone titers (Gregory, 1996).

The present study indicates that our specimen-handling protocols generated significant stress and may make data difficult to interpret because of their probable influence on testosterone titers. Without abandoning the convenience of utilizing incidentally-captured turtles, it is necessary to reduce the time from capture to sampling to a minimum. This would require a greater time commitment by the researchers, as it means being on board when the turtle is captured, although it would still be less effort than that required by relying upon directed-catch techniques.

Further studies in the same area as well as in other supposed foraging areas, including the Adriatic Sea (Argano et al., 1992), are necessary to obtain a useful map of Mediterranean sex ratio values at sea. At the same time it is necessary to know the primary sex ratios produced at the most important nesting grounds, in order both to understand the relationship with those at sea and to evaluate a possible human impact on sex ratio dynamics. It is clear that a good understanding of sex ratio dynamics can come only from data on age-class and geographic differences over the years.

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Female-Biased Sex Ratio of Juvenile Loggerhead Sea Turtles in Georgia

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Environmentally determined sex in sea turtles has the potential of producing a skewed sex ratio if mean incubation conditions in the nesting range of the species favor production of one sex and if a differential mortality rate does not compensate for this imbalance. Dodd (1988) questioned the validity of the assumption that an even sex ratio in logger-

head (*Caretta caretta*) hatchlings from a given nesting beach could be expected in any particular year. Based on sand and nest temperatures during a 3-year study, Camhi (1993) predicted male-biased hatchling production of loggerheads on Cumberland Island, Georgia. Henwood (1987) found males predominating in the Florida adult population, as did Wibbels et al. (1987a) for juveniles in Australia, while Wibbels et al. (1987b) reported a juvenile sex ratio skewed in favor of females from Florida and Chesapeake Bay. In a later paper, Wibbels et al. (1991) reported a preponderance of females in a Florida juvenile sample. Owens (1997) summarized juvenile sex ratio data derived from testosterone assays, some confirmed by laparoscopy, and sex ratios of all Atlantic population samples favored females. Females accounted for 66% of 103 stranded juveniles and adults along the Gulf Coast of Texas (Stabenau et al., 1996). Using a 10-year sample of stranded juveniles from Cumberland Island, Georgia, we address the question of sex ratio of the United States Western North Atlantic juvenile loggerhead population.

Determining population sex ratios in wide-ranging and migratory sea turtles is difficult because adult females usually skip one to several years of nesting (Dodd, 1988) and may not migrate or forage in the same habitats as do males (Henwood, 1987). Different behaviors and mortality risks of adults might result in a modified sex ratio over time or increased likelihood of sampling error. Attempting to define the Western Atlantic loggerhead hatchling population sex ratio would entail enormous effort to include the entire nesting range with defined temporal variation in sex determination and egg survival. We propose herein that the sex ratio of the juvenile population with presumably similar habits may better define the original hatchling sex ratio, or at least the sex ratio of pelagic-stage hatchlings. After maturity, the ratio could change given unequal mortality of the sexes, but the ratio of first breeders probably reflects the juvenile ratio.

Methods. — The 27 km beach of Cumberland Island, Camden County, Georgia, USA, was surveyed at least weekly and often daily for stranded, dead sea turtles from January 1986 through December 1995. In addition, National Park Service patrols and island visitors notified us of strandings, so that we necropsied or inspected most, if not all, stranded sea turtles. Curved carapace length (CCL) was measured to the nearest 0.5 cm from nuchal notch to tip of last marginal scute. Sex was determined by inspection of gonads (Wolke and George, 1981). Juveniles were defined by their state of gonadal differentiation: for females, ovarian follicles < 2 mm diameter; for males, no expansion (width < 1.5 cm) or elaboration of testes or complete elongation of the tail. Gonads were frequently missing in very rotten and shark- or vulture-mutilated carcasses. Gut contents were removed, washed, and dried or preserved in formalin for later analyses. Departures of observed sex ratios from expected values of 1:1 were tested using standard chi-square analysis.

Results. — During this study, 459 juvenile loggerheads were necropsied, but sex could not be determined in 139